α_1-Adrenoceptor-mediated negative inotropy of adrenaline in rat myocardium

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1. The effect of α- and β-adrenoceptor stimulation on isotonic contraction was investigated on right ventricular papillary muscles of the rat, stimulated at a rate of 0·5 Hz.

2. Adrenaline (0·5 μM) induced a slight but significant negative inotropic effect: shortening decreased from $0·137 \pm 0·058$ to $0·122 \pm 0·059$ muscle lengths (mean ± S.D.; −11%, $P < 0·0001$) and maximum shortening velocity from $2·9 \pm 1·2$ to $2·7 \pm 1·3$ muscle lengths s^{-1} (−7%, $P < 0·025$).

3. The negative inotropic effect of adrenaline was enhanced after blocking the β-adrenoceptors with 50 μM atenolol. On the other hand, exposure to adrenaline after blocking the α-adrenoceptors with 50 μM phentolamine resulted in an increase in shortening as well as in maximum shortening velocity.

4. Stimulation of the β-adrenoceptors with 0·5 μM isoprenaline caused marked positive inotropic effects, whereas stimulation of the α_1-adrenoceptors with 0·5 μM phenylephrine regularly resulted in a long-lasting decrease in shortening and maximum shortening velocity.

5. 1,2-Dioctanoyl-*sn*-glycerol (1,2-DOG) and adrenaline induced an activation of protein kinase C (PKC) with translocation of this enzyme from the cytosol to the sarcolemma.

6. Activation of PKC with 10 μM 1,2-DOG and 0·5 μM adrenaline was accompanied by a decrease in shortening and maximum shortening velocity. Inhibition of PKC with 0·1 μM staurosporine abolished the negative inotropic effect of adrenaline.

7. From these results we conclude that a low dose of adrenaline stimulates not only β- but also α-adrenoceptors and that the observed negative inotropic effect of adrenaline is mediated by α_1-adrenoceptors, linked to the diacylglycerol–PKC signal transduction pathway.

The sympathetic nervous system modulates cardiac contraction via α- and β-adrenoceptors. The activation of the β-adrenoceptors results principally in an increase in cAMP, whereas the α-adrenoceptors are linked to two signal transducing pathways, i.e. inositol trisphosphate and diacylglycerol (Berridge, 1984).

The modulation of cardiac contractility by α_1-adrenoceptors has been reviewed recently in detail (Fedida, 1993; Terzic, Pucéat, Vassort & Vogel, 1993). Positive inotropic effects of α_1-adrenoceptor stimulation have been well established, although significant differences exist from species to species. On the other hand, particularly in the rat, α_1-adrenoceptors also mediate negative inotropic effects in the isolated perfused heart (Watanabe, Hathaway, Besch, Farmer & Harris, 1977), as well as in the papillary muscle (Skomedal, Osnes & Øye, 1982; Otani, Otani & Das, 1988; Endou, Hattori, Those & Kanno, 1991; Reichel, 1996) and also in isolated myocytes (Capogrossi, Kachadorian, Gambassi, Spurgeon & Lakatta, 1991; Gambassi, Spurgeon, Lakatta, Blank & Capogrossi, 1992; Terzic, Pucéat, Clément, Scamps & Vassort, 1992). In all these studies, however, the α_1-adrenoceptors were activated with the synthetic α_1-adrenoceptor stimulant, phenylephrine. Furthermore, the observed negative inotropy was only transient and was followed regularly by a long-lasting positive inotropy.

The mechanisms behind the α_1-adrenoceptor-mediated negative inotropy are still unclear. There is some evidence that protein kinase C (PKC) may be involved, since phorbol esters as well as 1,2-dioctanoylglycerol can produce negative inotropic effects in whole-heart preparations (Yuan, Sunahara & Sen, 1987), papillary muscles (Otani, Hara, Xun-Ting, Omori & Inagaki, 1992), atria (Teutsch, Weible & Siess, 1987) and isolated myocytes (Capogrossi *et al.* 1990).

The present study was performed in order to investigate whether an α_1-adrenoceptor-mediated negative inotropy can also be demonstrated with physiological doses of adrenaline. Furthermore, we attempted to elucidate the involvement of PKC in the α_1-adrenoceptor-mediated negative inotropy.

METHODS

Papillary muscle preparation and measurement of shortening

Forty male Wistar rats (body weight, 399 ± 82 g) were anaesthetized with diethyl ether and the hearts excised rapidly and transferred to a dissection bath. The right ventricle was opened carefully and both ends of a papillary muscle were fixed to the ventricular wall with thin glass capillaries prior to the dissection of the preparation. With this procedure it was possible to dissect even very thin papillary muscles without any stretching of the preparations. The papillary muscles (length, 2·46 ± 0·70 mm; diameter, 0·45 ± 0·18 mm) were transferred to a tissue bath of 5 ml volume.

The glass capillary at one end of the preparation was connected to a fixed hook and the capillary at the other end to a lever attached to a moving coil amperemeter. By this means it was possible to adjust the isotonic load exactly by varying the current flowing through the amperemeter. The length and diameter of the preparations were determined with a microscope containing a graduated ocular. The cross-sectional area of the preparations was calculated assuming a circular cross-section and the isotonic load was adjusted with the amperemeter to a value of 5 mN mm^{-2}. By placing the movable lever between a photocell and a light source it was possible to measure the shortening of the preparations accurately. The output of the photocell, i.e. change in muscle length, and its differential (Biotronex BL 622; cut-off frequency, 320 Hz), shortening velocity, were displayed on an oscilloscope (Tectronix) and registered continuously with a direct recorder (Hellige) at low paper speed (5 mm min^{-1}). At certain intervals both signals were registered additionally with a UV recorder (Honeywell) at high paper speed (100 mm s^{-1}).

Experimental protocol

The tissue bath was perfused at a rate of 6 ml min^{-1} with a modified Tyrode solution having the following composition (mM): NaCl, 130; KCl, 4·1; $CaCl_2$, 1·1; $MgCl_2$, 1·5; $NaHCO_3$, 20; NaH_2PO_4, 1·2; glucose, 25. The solution was gassed with 95% O_2–5% CO_2, yielding a pH value of 7·36 ± 0·04, and the temperature was kept at 35 °C. The preparations were stimulated at a rate of 0·5 Hz with an electronic stimulator (Tönnies, Freiburg, Germany) through platinum field electrodes. Rectangular impulses of 2 ms duration were applied at 1·2 times the threshold voltage through an isolation unit.

In five preparations shortening as well as shortening velocity were measured over a period of 450 min without any pharmacological intervention. Both parameters declined with time (Fig. 1). Pilot experiments have shown that in the early stage of the experiments, i.e. at times when the preparations still vigorously shorten, exposure to adrenaline frequently caused spontaneous activity. Since in later stages of the experiments adrenaline-induced spontaneous activity was not observed, in further experiments all preparations were equilibrated for at least 90 min before the commencement of the experiments.

The following compounds were used: adrenaline hydrochloride (Suprarenin®, Hoechst), atenolol (Sigma), phentolamine methanesulphonate (Regitin®, Ciba), L-phenylephrine hydrochloride (Sigma), (−)-isoproterenol (isoprenaline) hydrochloride (Sigma), staurosporine (Sigma) and 1,2-dioctanoyl-*sn*-glycerol (1,2-DOG) (Sigma). Staurosporine and 1,2-DOG were dissolved in dimethyl sulphoxide (DMSO) and all the other compounds were dissolved in ungassed Tyrode solution. The compounds were administered with motor-driven syringes (Unita II, Braun, Melsungen, Germany) into the perfusion line immediately in front of the tissue bath. In order to ensure protection against light, the syringes, as well as the connecting tubes, were encased in light-resistant black foil. The concentrations of the different compounds in the syringes were chosen such that the rate of infusion never exceeded 0·075 ml min^{-1}. The preparations were exposed to the compounds under test until a new steady state was achieved, which generally happened after 5 to 10 min. Thereafter, washout periods of at least 20 min duration were maintained. Since 1,2-DOG as well as staurosporine had to be dissolved in DMSO, these compounds were applied at the end of the experiments.

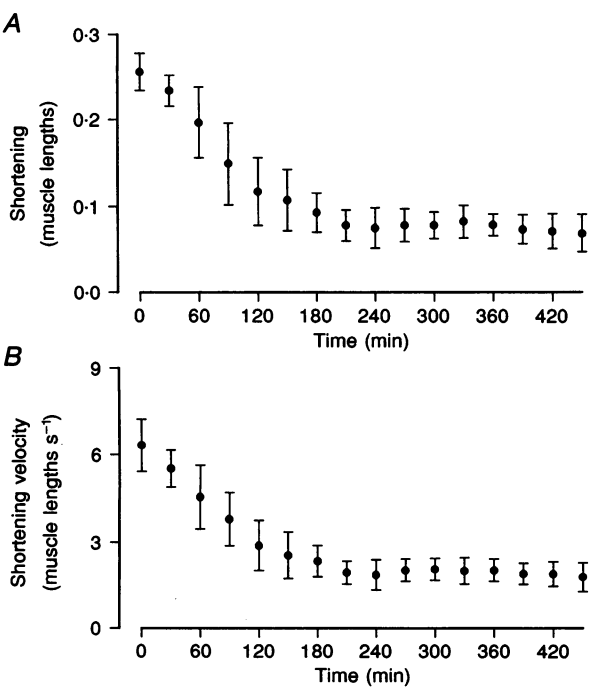

Figure 1. Time courses of shortening and maximum shortening velocity measured on five right ventricular papillary muscles over a period of 450 min

A, shortening; *B*, maximum shortening velocity. The preparations were superfused with Tyrode solution and stimulation rate was 0·5 Hz. Both variables declined during the first 2 h and subsequently remained relatively constant.

In papillary muscles from three hearts the activity of PKC was measured in the membrane-bound and cytosolic fractions during exposure to 1,2-DOG. In six hearts it was possible to dissect two suitable papillary muscles from each. In these experiments the membrane-bound and cytosolic activity of PKC was measured under control conditions in one papillary muscle and during the action of adrenaline in the other.

PKC assay

Muscle preparations were transferred to 200 μl of extraction buffer (20 mM Tris (pH 7·5), 0·5 mM EDTA, 0·5 mM EGTA, 0·5% Triton X-100 and 25 μg ml^{-1} each of aprotinin and leupeptin) and homogenized using a Dounce homogenizer. After 15 min incubation at 20 °C the samples were centrifuged for 30 min at 300 000 *g*. The supernatant was composed of cytosol and the pellet was membrane material. Of the cytosol, 25 μl were added to 20 μl of extraction buffer supplemented with 10 mM β-mercaptoethanol and 200 mM NaCl and 5 μl of a lipid preparation consisting of phosphatidyl serine and phorbol ester in Triton X-100 (reaction buffer as supplied by Gibco). Pellets were resuspended in 20 μl of reaction buffer as above. To control the specificity of the reaction, a PKC inhibitor (a pseudosubstrate consisting of the amino acids 19–36 of PKC) was added to some of each sample. After 20 min incubation at 20 °C, allowing the inhibitor and the phospholipids to bind, the reaction was initiated by addition of the PKC substrate peptide and 10 μCi [^{32}P]γATP per sample. The samples were then incubated for 5 min at 30 °C. The reaction was terminated by filtration of aliquots through nitrocellulose filters. The filters were washed twice in 1% H_3PO_4, twice in H_2O and radioactivity bound to the filters was determined by liquid scintillation counting. Binding of the phosphorylated substrate reflects the activity of the kinase.

Statistics

All given values are expressed as means ± S.D. Student's paired *t* test was used to compare values measured before and after administration of the compound under test. Statistically significant differences were assumed at $P < 0·05$.

RESULTS

The effect of 0·5 μM adrenaline on shortening and maximum shortening velocity was investigated in twenty-three experiments on fifteen preparations. A tracing of a representative experiment is shown in Fig. 2*A*. Shortening as well as shortening velocity declined during exposure to adrenaline. In seven experiments a biphasic reaction was found, i.e. both parameters decreased at first and recovered between the fifth and tenth minute, and in three experiments even exceeded the values measured under control conditions. In Fig. 2*B* steady-state values of shortening under adrenaline are plotted against the values of shortening under control conditions. On average, shortening declined from 0·137 ± 0·058 to 0·122 ± 0·059 muscle lengths (−11%, $P < 0·0001$) and maximum shortening velocity declined from 2·9 ± 1·2 to 2·7 ± 1·3 muscle lengths s^{-1} (−7%, $P < 0·025$, Fig. 2*C*).

In nineteen experiments on eight preparations the β-adrenoceptors were blocked with 50 μM atenolol prior to the application of 0·5 μM adrenaline. Under these conditions the negative inotropic effect of adrenaline was definitely

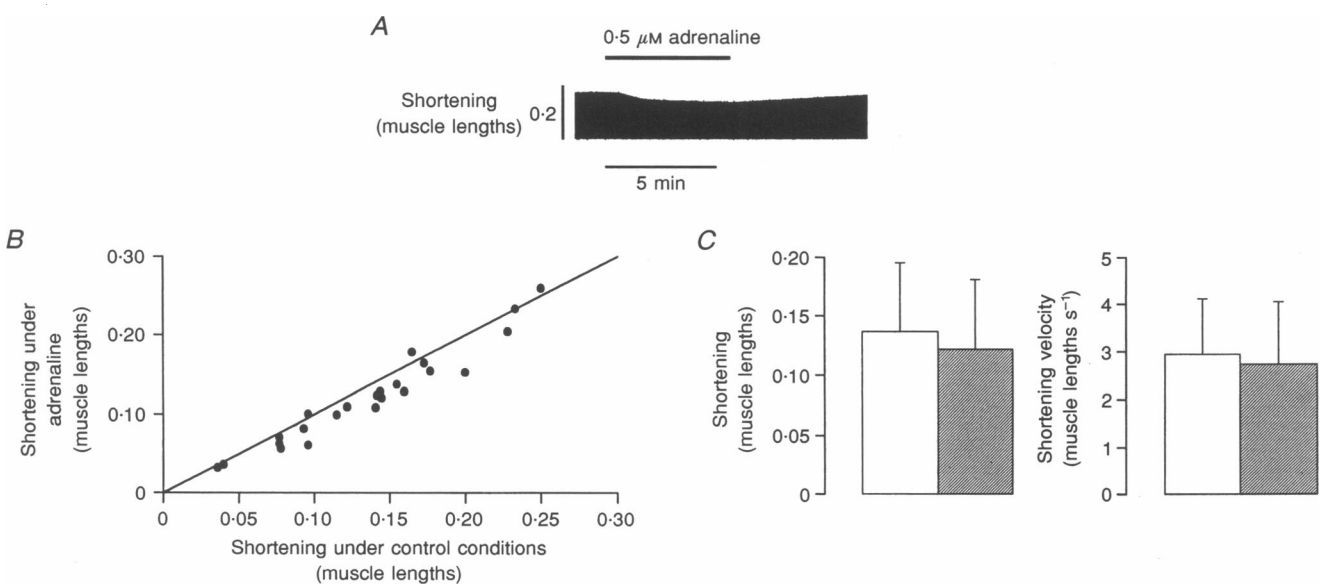

Figure 2. Effect of 0·5 μM adrenaline on isotonic contraction of right ventricular papillary muscles

A, representative original tracing; application of adrenaline is indicated by the horizontal bar. *B*, plot of shortening values measured in the steady state obtained during exposure to adrenaline (ordinate) against control values of shortening measured under Tyrode solution (abscissa). The straight line indicates the line of unity. *C*, mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured before (□) and during (▩) the application of adrenaline.

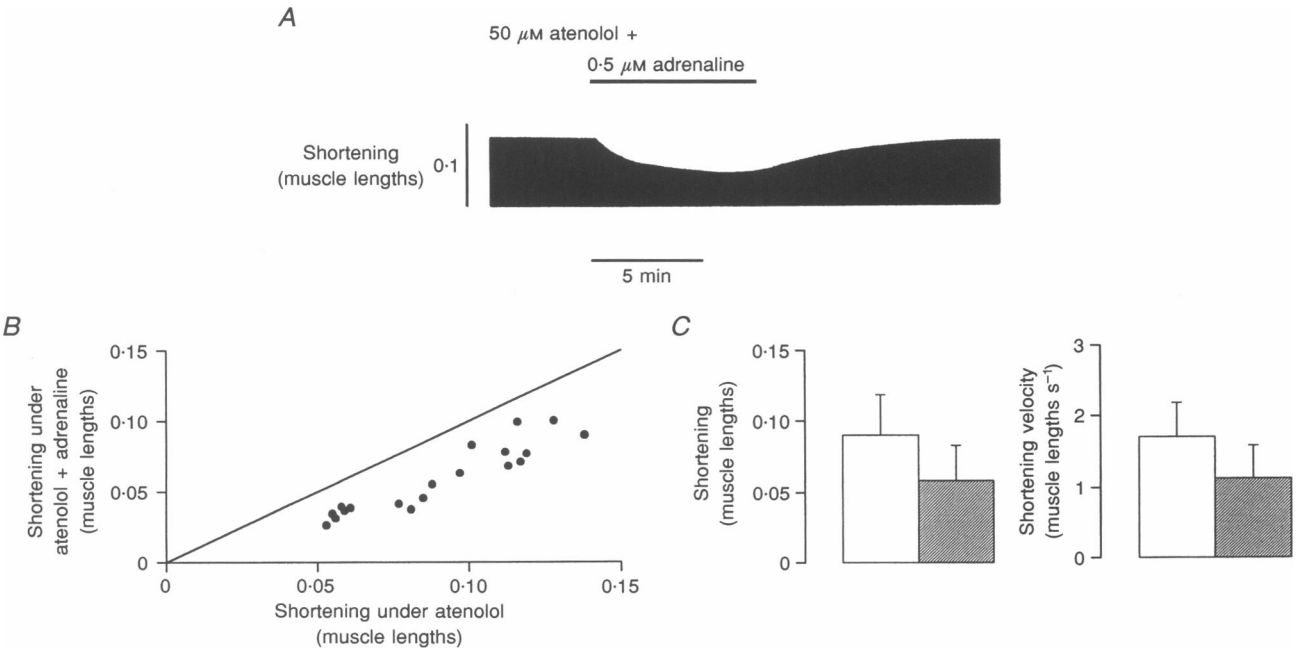

Figure 3. Effect of 0·5 μM adrenaline on isotonic contraction after blocking the β-adrenoceptors with 50 μM atenolol

A, representative original tracing; application of adrenaline is indicated by the horizontal bar. *B*, shortening measured under adrenaline plus atenolol (ordinate) plotted against shortening measured under atenolol alone (abscissa). All values clearly lie below the line of unity. *C*, mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured under atenolol (□) and under atenolol plus adrenaline (▨).

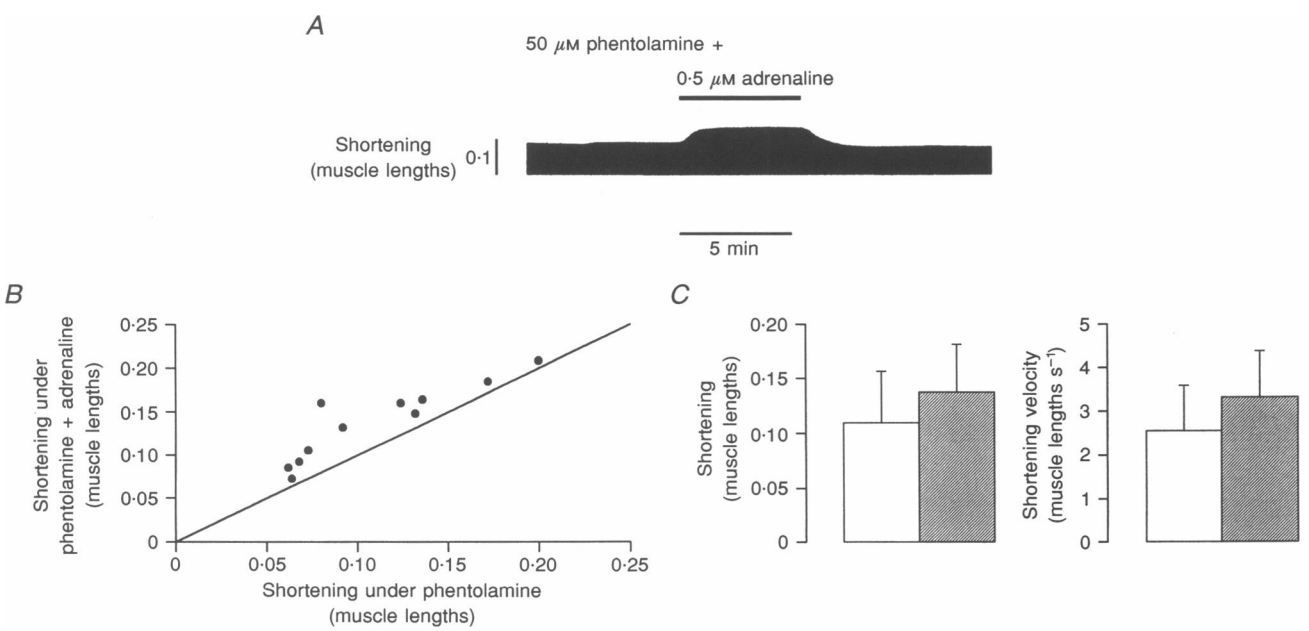

Figure 4. Effect of 0·5 μM adrenaline on isotonic contraction after blocking the α-adrenoceptors with 50 μM phentolamine

A, representative original tracing; exposure to adrenaline is indicated by the horizontal bar. *B*, plot of shortening under adrenaline plus phentolamine (ordinate) against shortening under phentolamine (abscissa). All values lie above the line of unity. *C*, mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured under phentolamine (□) and under phentolamine plus adrenaline (▨).

enhanced (Fig. 3*A*). Shortening and shortening velocity decreased in all experiments without any recovery and the amount of negative inotropy was independent of shortening under control conditions (Fig. 3*B*). Mean values of shortening decreased from $0{\cdot}090 \pm 0{\cdot}028$ to $0{\cdot}058 \pm 0{\cdot}024$ muscle lengths (−36%, $P < 0{\cdot}0001$) and mean values of maximum shortening velocity decreased from $1{\cdot}7 \pm 0{\cdot}5$ to $1{\cdot}1 \pm 0{\cdot}5$ muscle lengths s^{-1} (−35%, $P < 0{\cdot}0001$, Fig. 3*C*).

In a further series of eleven experiments on six preparations the α-adrenoceptors were blocked with 50 μM phentolamine prior to the application of 0·5 μM adrenaline (Fig. 4). After blocking the α-adrenoceptors, adrenaline consistently exerted a positive inotropic effect (Fig. 4*A*), which was independent of the contractile state under control conditions (Fig. 4*B*). Shortening increased, on average, from $0{\cdot}109 \pm 0{\cdot}047$ to $0{\cdot}137 \pm 0{\cdot}044$ muscle lengths (+26%, $P < 0{\cdot}005$) and maximum shortening velocity increased from $2{\cdot}5 \pm 1{\cdot}0$ to $3{\cdot}3 \pm 1{\cdot}1$ muscle lengths s^{-1} (+32%, $P < 0{\cdot}0001$, Fig. 4*C*).

The results of the experiments with adrenoceptor-blocking agents suggest that the negative inotropic effect of adrenaline is mediated by α-adrenoceptors. This notion was strengthened by a further eight experiments on four papillary muscles, in which the α_1-adrenoceptors were stimulated with 0·5 μM phenylephrine. This low dose of phenylephrine exerted a small, but sustained and significant negative inotropic effect (Fig. 5). Shortening decreased from $0{\cdot}084 \pm 0{\cdot}021$ to $0{\cdot}071 \pm 0{\cdot}020$ muscle lengths (−15%, $P < 0{\cdot}0005$) and maximum shortening velocity decreased from $2{\cdot}0 \pm 0{\cdot}5$ to $1{\cdot}6 \pm 0{\cdot}5$ muscle lengths s^{-1} (−20%, $P < 0{\cdot}005$). On the other hand, stimulation of the β-adrenoceptors with 0·5 μM isoprenaline (8 experiments on 2 papillary muscles) resulted in a significant increase in shortening from $0{\cdot}110 \pm 0{\cdot}044$ to $0{\cdot}153 \pm 0{\cdot}043$ muscle lengths (+39%, $P < 0{\cdot}01$) and a significant increase in maximum shortening velocity from $2{\cdot}4 \pm 0{\cdot}8$ to $3{\cdot}9 \pm 1{\cdot}1$ muscle lengths s^{-1} (+63%, $P < 0{\cdot}005$, Fig. 6).

It has been shown that α-adrenoceptors are linked to several intracellular signalling molecules, in particular inositol trisphosphate and diacylglycerol, causing an increase in PKC activity. The possible role of PKC in mediating the catecholamine-induced negative inotropy was examined either by stimulating this kinase with 1,2-DOG or by blocking it with staurosporine. Initially, it was established that the vehicle for both substances, i.e. DMSO, exerts no effects on shortening and shortening velocity at the rate of infusion used in these experiments (0·02 ml min^{-1}).

PKC activity was stimulated with 10 μM 1,2-DOG in ten experiments on five papillary muscles (Fig. 7). Under 1,2-DOG stimulation shortening decreased from $0{\cdot}062 \pm 0{\cdot}021$ to $0{\cdot}041 \pm 0{\cdot}016$ muscle lengths (−34%, $P < 0{\cdot}0001$) and maximum shortening velocity decreased from $1{\cdot}4 \pm 0{\cdot}4$

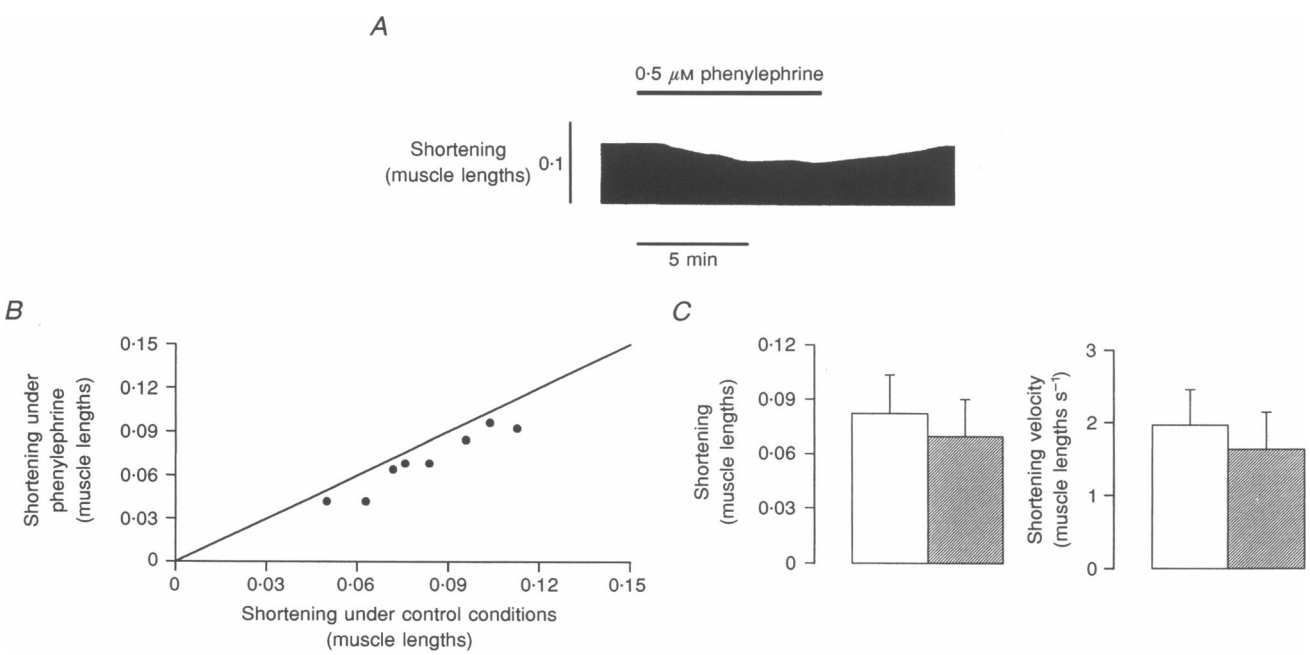

Figure 5. Stimulation of α_1-adrenoceptors with 0·5 μM phenylephrine causes a decrease in shortening and in maximum shortening velocity

A, representative original tracing; application of phenylephrine is indicated by the horizontal bar. *B*, shortening under phenylephrine (ordinate) is plotted against shortening under Tyrode solution (abscissa). All the values lie below the line of unity. *C*, mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured before (□) and during (▨) exposure to phenylephrine.

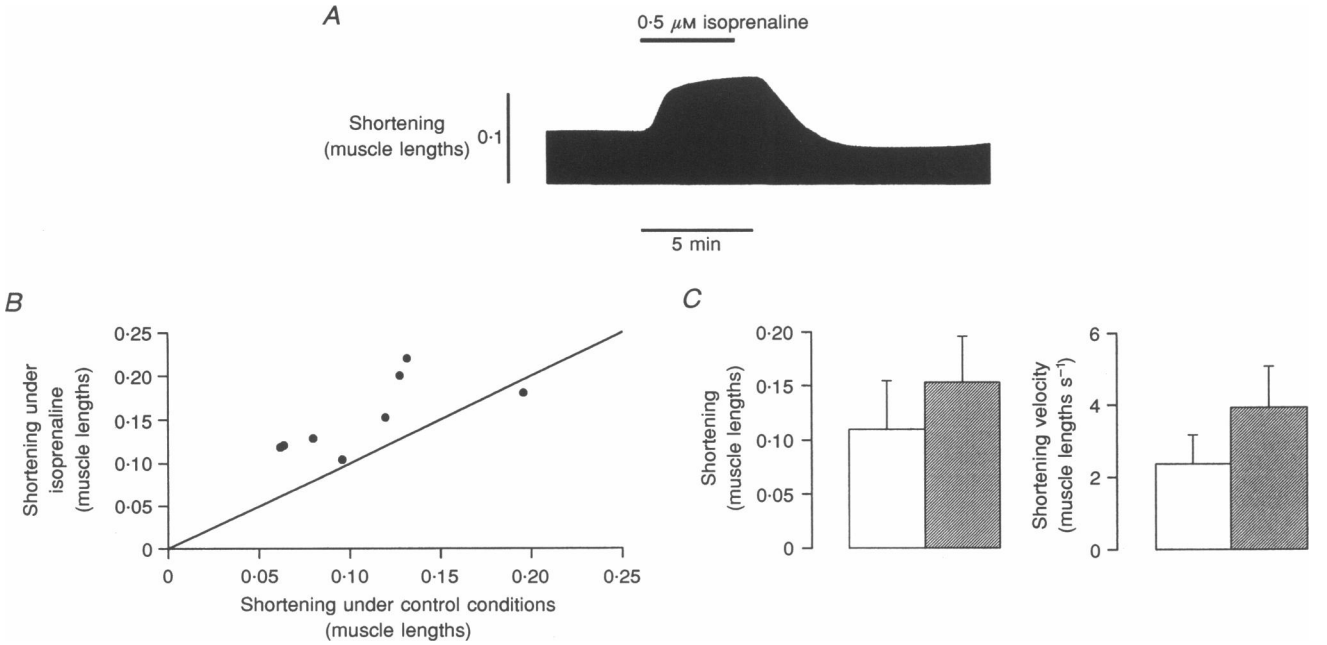

Figure 6. Stimulation of β-adrenoceptors with 0·5 μM isoprenaline results in an increase in shortening and in maximum shortening velocity

A, representative original tracing; exposure to isoprenaline is indicated by the horizontal bar. *B*, plot of shortening under isoprenaline (ordinate) against shortening under control conditions (abscissa); also shown is the line of unity. *C*, mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured under control conditions (□) and during the application of isoprenaline (▨).

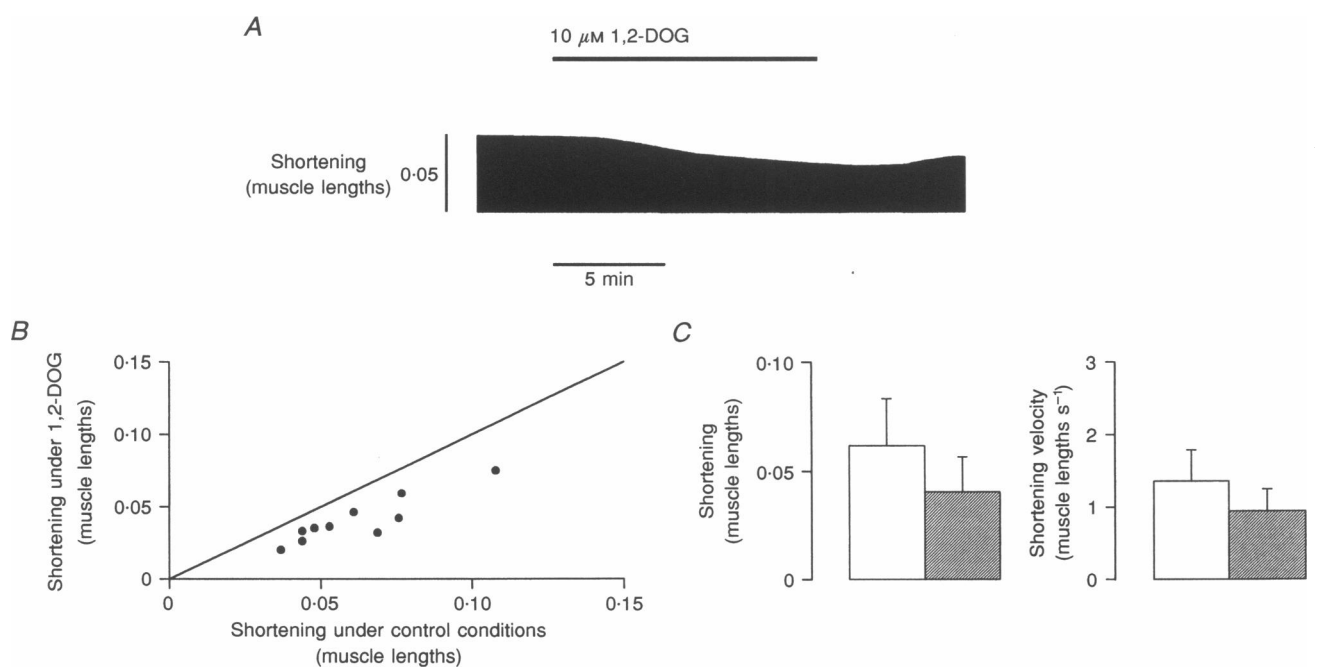

Figure 7. Effect of 10 μM 1,2-dioctanoyl-*sn*-glycerol on isotonic contraction

A, representative original tracing; application of 1,2-DOG is indicated by the horizontal bar. *B*, plot of shortening under 1,2-DOG (ordinate) against shortening under control conditions (abscissa). Exposure to 1,2-DOG regularly results in a decrease in shortening, so that all the values lie below the line of unity. *C*, mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured under control conditions (□) and during exposure to 1,2-DOG (▨).

to $1{\cdot}0 \pm 0{\cdot}3$ muscle lengths s^{-1} (−29%, $P < 0{\cdot}001$). The effect of 1,2-DOG, unlike that of staurosporine, was reversible with washout of the compound within 30 min.

The experiments with staurosporine were performed on four papillary muscles. First the β-adrenoceptors were blocked with 50 μM atenolol and the preparations superfused with 0·02 ml min^{-1} DMSO. The effect of 0·5 μM adrenaline was examined in nine experiments prior to, and in sixteen experiments after, superfusion for 2 h with 0·1 μM staurosporine. The results of these experiments are shown in Fig. 8. Prior to staurosporine, adrenaline induced a significant negative inotropic effect. Shortening decreased from $0{\cdot}092 \pm 0{\cdot}030$ to $0{\cdot}064 \pm 0{\cdot}028$ muscle lengths (−30%, $P < 0{\cdot}0001$) and maximum shortening velocity decreased from $1{\cdot}8 \pm 0{\cdot}5$ to $1{\cdot}3 \pm 0{\cdot}5$ muscle lengths s^{-1} (−28%, $P < 0{\cdot}0005$). Under staurosporine, however, both parameters remained unchanged during the application of adrenaline (shortening, $0{\cdot}089 \pm 0{\cdot}038$ *vs.* $0{\cdot}088 \pm 0{\cdot}035$ muscle lengths; maximum shortening velocity, $1{\cdot}7 \pm 0{\cdot}9$ *vs.* $1{\cdot}7 \pm 0{\cdot}8$ muscle lengths s^{-1}).

Our experiments with staurosporine as well as with 1,2-DOG indicate that the negative inotropic effect, observed during the stimulation of α_1-adrenoceptors, is probably mediated by the diacylglycerol pathway via PKC. Evidence for this hypothesis may be drawn from our experiments in which the activity of PKC was measured. In three papillary muscles the activity of PKC was measured during exposure to 10 μM 1,2-DOG. In another series of experiments we excised from each of six hearts two separate papillary muscles; one preparation was given no pharmacological treatment and the other preparation from the same heart was exposed to 0·5 μM adrenaline plus 50 μM atenolol. All

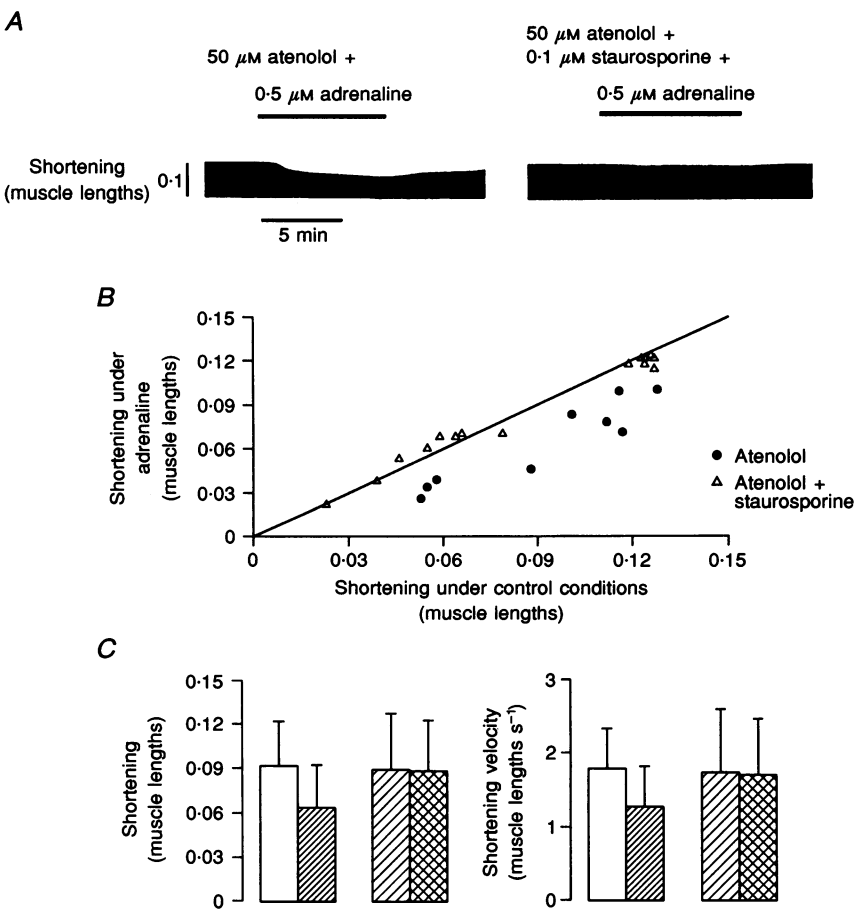

Figure 8. Effect of staurosporine on adrenaline-induced negative inotropy

A, representative original tracings showing the effect of 0·5 μM adrenaline under β-blockade (50 μM atenolol) prior to (left) and during exposure to 0·1 μM staurosporine (right). Application of adrenaline is indicated by the horizontal bars. *B*, plot of shortening measured under adrenaline, prior to and after exposure to staurosporine (ordinate) against shortening measured under the respective control conditions, i.e. under atenolol and under atenolol plus staurosporine (abscissa). The negative inotropic effect of adrenaline, observed after blocking the β-adrenoceptors with atenolol, is completely prevented after exposure to staurosporine. *C*, effects of adrenaline on mean values and standard deviation of shortening (left) and maximum shortening velocity (right), measured under β-blockade prior to and after exposure to staurosporine. Atenolol, □; atenolol + adrenaline, ▨; atenolol + staurosporine, ▨; atenolol + staurosporine + adrenaline, ▩.

preparations were paced as previously at 0·5 Hz. The papillary muscles, exposed either to 1,2-DOG or to adrenaline, were quickly transferred from the tissue bath to the extraction buffer at that moment when isotonic contraction had reached a new steady state; the untreated papillary muscles were transferred at identical times. An increase in PKC activity is thought to be accompanied by the translocation of this enzyme from the cytosol to the sarcolemma (Henrich & Simpson, 1988). The activity of PKC was determined in the particulate and the cytosolic fraction from unstimulated and stimulated muscles. The activity was measured by incorporation of [^{32}P] from [^{32}P]γATP into a PKC substrate peptide. The activity measured before stimulation was taken as 100%, and the percentage increase in activity, measured during stimulation, corresponds directly to the amount of activated PKC.

Under 10 μM 1,2-DOG, shortening decreased in the three preparations by 23, 27 and 33%. At the same time the activity of PKC increased in the particulate fraction by 54, 239 and 158%. Due to technical difficulties the determination of PKC activity in the cytosolic fraction was only possible in two preparations and was found to change by +35 and −15%, respectively. In the six papillary muscles exposed to 0·5 μM adrenaline shortening decreased by 29 ± 14% ($P < 0·005$); the simultaneously measured activities of PKC are summarized in Fig. 9. Under control conditions, i.e. without adrenaline, no substantial increase in activity appeared during stimulation: +21 ± 17% in the particulate fraction and +42 ± 48% in the cytosolic fraction. Exposure to 0·5 μM adrenaline, however, resulted in a significant increase in activity in the particulate fraction by 169 ± 106% ($P < 0·025$), whereas the activity in the cytosolic fraction remained almost constant (+16 ± 39%, not significant). The increase in activity in the particulate fraction is specific to PKC since it could be prevented by a pseudosubstrate of PKC, consisting of the amino acids 19–36 of PKC that act as a specific PKC inhibitor (right columns in Fig. 9).

DISCUSSION

The present results clearly demonstrate that in the isotonically beating right ventricular papillary muscle of the rat a low dose of adrenaline induces a moderate negative inotropic effect. Since this effect is enhanced during the blockade of the β-adrenoceptors and abolished by blocking the α-adrenoceptors, it must be concluded that the negative inotropic effect of adrenaline is mediated by α-adrenoceptors. This is supported by our finding that the application of a low dose of the α_1-adrenergic agonist phenylephrine also consistently yields a small but long-lasting negative inotropy.

In the present experiments the preparations were exposed to the different compounds after an equilibration period of at least 90 min, i.e. at times when shortening declined to about 60% of the initial value (Fig. 1). The long equilibration period must be observed since pilot experiments have shown that exposure to adrenaline during the early stages of the experiments, i.e. at times when the preparations still vigorously shorten, regularly caused an

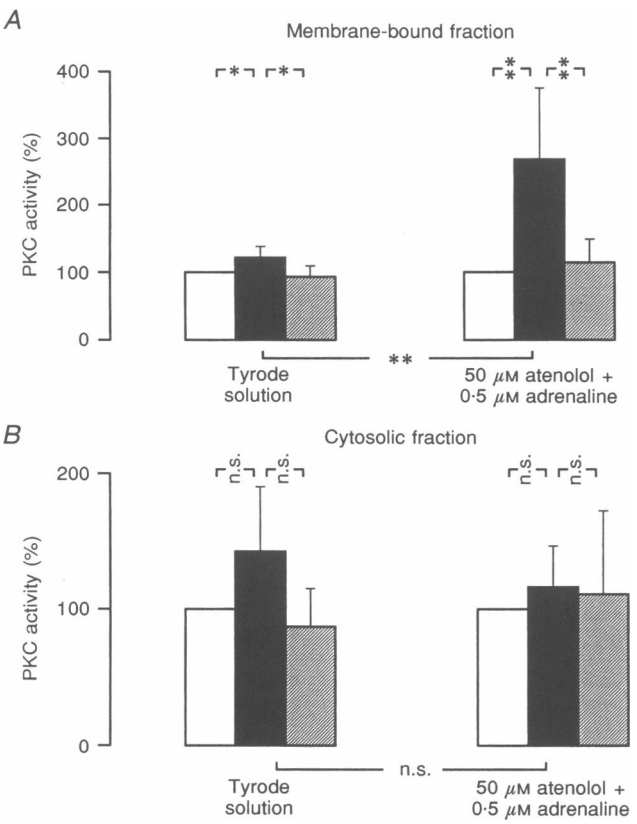

Figure 9. Effect of adrenaline on PKC activity

Relative PKC activity was measured in membrane-bound fractions (*A*) and in cytosolic fractions (*B*) of isotonically beating right ventricular papillary muscles under control conditions (Tyrode solution) and during exposure to 0·5 μM adrenaline after blocking the β-adrenoceptors with 50 μM atenolol. Non-specific activity in samples without the addition of cofactors has been set to 100% in each case. Under control conditions, only a negligible increase in activity is detectable in either the membrane-bound or cytosolic fraction. Superfusion with adrenaline, however, yielded a significant increase in activity but only in the membrane-bound fraction, indicating translocation of PKC from the cytosol into the sarcolemma. The increase in phosphorylation is specific for PKC, since it is prevented by a pseudosubstrate of PKC, consisting of the amino acids 19–36 of PKC which act as a specific inhibitor of PKC. □, non-specific activity; ■, activity during PKC stimulation; ▨, activity after inhibition of PKC. * $P < 0·05$; ** $P < 0·025$; n.s., not significant.

initial decrease in shortening which, however, was frequently followed by spontaneous activity. Nevertheless, dependence of the observed pharmacological effects upon initial shortening is unlikely since we observed that initial muscle shortening and pharmacological effects do not correlate with each other as can be derived from Figs 2*B*–8*B*. Further pilot experiments, performed on two papillary muscles, have shown that the negative inotropic effect of adrenaline is not specific to isotonic contraction, but is also evident in the isometrically beating papillary muscle. Furthermore, the negative inotropic effect of adrenaline is not restricted to a stimulation rate of 0·5 Hz, since additional pilot experiments at 0·2, 1 and 2 Hz yielded qualitatively similar effects.

Ligand-binding studies have proved the existence of α_1-adrenoceptors in the myocardium (Steinberg & Bilezikian, 1982). The density of myocardial α_1-adrenoceptors varies with species and the myocardium of the rat in particular possesses a high number of α_1-adrenoceptor binding sites (Mukherjee *et al.* 1983; Endoh, Hiramoto, Ishihata, Takanashi & Inui, 1991). According to most studies the stimulation of myocardial α_1-adrenoceptors results in a positive inotropic effect (for review see Fedida, 1993; Terzic *et al.* 1993). In rat myocardium, soon after exposure to phenylephrine and prior to the positive inotropic effect, a small and transient negative inotropy has been observed (Watanabe *et al.* 1977; Skomedal *et al.* 1982; Otani *et al.* 1988; Capogrossi *et al.* 1991; Endou *et al.* 1991; Gambassi *et al.* 1992; Terzic *et al.* 1992; Reichel, 1996). Since phenylephrine is known to activate not only α- but also β-adrenoceptors, in the above studies phenylephrine has been applied in combination with β-adrenoceptor-blocking agents, hence the observed changes in inotropy must be a result of α_1-adrenergic stimulation.

Recently it has been shown that myocardial α_1-adrenoceptors can be subdivided into at least three subtypes, i.e. α_{1A}, α_{1B} and α_{1D} (Graham, Perez, Hwa & Piascik, 1996), possibly linked to different signal transduction pathways and effector systems. However, conflicting results have been reported with regard to the allocation of distinct physiological effects to the respective receptor subtypes. In rat ventricular myocardium (Michel, Hanft & Gross, 1994) and in rabbit ventricular myocardium (Takanashi, Norota & Endoh, 1991) the positive inotropic effect of adrenaline was found to be mediated mainly by α_{1B}-adrenoceptors. In another study on rat myocardium the positive inotropic effect of phenylephrine was antagonized by blocking the α_{1A}-adrenoceptors with WB 4101 (2-([2,6-dimethoxyphenoxyethyl]aminomethyl)-1,4-benzodioxane), but not affected by the α_{1B} antagonist, chlorethylclonidine (Nagashima, 1994). Furthermore, in rabbit myocardium the accumulation of [^{3}H]inositol 1,4,5-trisphosphate induced by phenylephrine was abolished after blocking the α_{1B}-adrenoceptors (Yang & Endoh, 1994), whereas in rat myocardium phosphoinositide hydrolysis induced by phenylephrine was inhibited by WB 4101, i.e. by blocking the α_{1A}-adrenoceptors (Nagashima, 1994).

The biphasic response of the mechanical activity to phenylephrine observed in the above-mentioned studies demonstrates that, even in the same experiment, opposite effects due to α-adrenoceptor stimulation may occur. These opposite effects might be explained by the assumption that the α_1-adrenoceptor subtypes are linked to different effector systems and that the individual receptor subtypes have different sensitivities to α_1-adrenoceptor agonists. α_1-Adrenoceptor subtypes with a high sensitivity to agonists could be stimulated at a low concentration of the agonist, exerting a distinct effect. Stimulation of subtypes with a low sensitivity requires higher concentrations and exerts an opposite effect, which may supersede the effects observed at low concentrations. After the addition of phenylephrine to the tissue bath the concentration increases gradually, so that soon after exposure a low concentration takes effect and later a high one. In fact, in all studies in which a biphasic response has been observed, the final concentration of phenylephrine was at least 20 times higher than in the present experiments. Therefore, the observed opposite effects of phenylephrine on myocardial contractility could simply be explained by different concentrations of phenylephrine during the same experiment. In the present experiments the papillary muscles have been exposed to 0·5 μM phenylephrine, which is a low concentration, and we never observed any positive inotropic effects but consistently a small and long-lasting negative inotropy (Fig. 5).

These considerations are also valid for the explanation of the effects of adrenaline observed in the present experiments. Exposure to 0·5 μM adrenaline resulted, on average, in a decrease in shortening by 11% (Fig. 2). At this low concentration the α-adrenoceptor-mediated negative inotropy obviously prevails over the β-adrenoceptor-mediated positive inotropy. The pure α-adrenoceptor-mediated effects of adrenaline appeared after blocking the β-adrenoceptors with atenolol (Fig. 3). Under these conditions shortening decreased by 36%, indicating that the negative inotropic effects of adrenaline have been masked, in part, by the simultaneous activation of β-adrenoceptors. On the other hand, after blocking the α-adrenoceptors with phentolamine, the pure β-adrenoceptor-mediated effects of adrenaline are forthcoming and shortening increased by 26% (Fig. 4). Higher doses of adrenaline (up to 10 μM) were applied in pilot experiments; they regularly caused positive inotropic effects only, i.e. at high adrenaline concentrations the β-adrenoceptor-mediated positive inotropic effect supersedes the α-adrenoceptor-mediated negative inotropy.

In the present experiments not only low doses of adrenaline and phenylephrine, but also exposure to 10 μM 1,2-DOG, exerted a significant decrease in shortening and in the maximum shortening velocity (Fig. 7). This demonstrates that the negative inotropic effect is probably mediated via PKC, which could be shown to be increased during exposure to 1,2-DOG. We also measured the cytosolic and membrane-bound activity of this kinase under control conditions and during exposure to adrenaline in papillary muscles from the

same hearts. Under control conditions a low activity of the membrane-bound fraction of PKC was measured, which increased significantly during exposure to adrenaline, whereas in the cytosol no nominal value of activity could be detected, either under control conditions or during exposure to adrenaline (Fig. 9). Therefore, in the present experiments the negative inotropic effect of adrenaline is accompanied by an increase in PKC activity.

However, conflicting results have been reported with regard to the effects of PKC: activation of this enzyme was found to increase myocardial contractility (Teutsch *et al.* 1987; Otani *et al.* 1988), not to be involved in α_1-adrenoceptor-mediated positive inotropic effects (Endou *et al.* 1991), to antagonize α-adrenoceptor-mediated positive inotropy (Kushida, Hiramoto, Satoh & Endoh, 1988) and to exert negative inotropic effects (Yuan *et al.* 1987; Capogrossi *et al.* 1990). These discrepancies in results may be attributed to the existence of various subtypes of PKC (Ohno, Akita, Konno, Imajoh & Suzuki, 1988) as well as to differences in both receptor distribution and intracellular coupling processes from one species to another (Endoh *et al.* 1991).

The assumption that the negative inotropic effect of adrenaline is mediated by PKC is supported by our experiments with staurosporine. This potent inhibitor of phospholipid and calcium-dependent PKC completely blocked the negative inotropic effects of adrenaline (Fig. 8). Total inhibition of PKC occurs only after a relatively long time of exposure to staurosporine, according to our experience after at least 2 h. The observation that staurosporine was not able to antagonize the negative inotropic effects of phorbol esters (Siems & Brasch, 1995) can therefore easily be explained by the fact that, in this study, the preparations were exposed to staurosporine for only 30 min.

On the basis of the present experiments, there is no doubt that activation of PKC is involved in α_1-adrenoceptor-mediated negative inotropy. In the myocardium, however, PKC phosphorylates different proteins in the sarcolemma (Iwasa & Hosey, 1984) as well as in the sarcoplasmic reticulum (Limas, 1980; Movesian, Nishikawa & Adelstein, 1984) and in the myofilaments (Katoh, Wise & Kuo, 1983). Additionally, activation of PKC causes cytosolic alkalinization via activation of the Na^+–H^+ exchanger (Grinstein & Rothstein, 1986; Gambassi *et al.* 1992), which in turn results in an increase in intracellular Na^+, a substantial determinant of Na^+–Ca^{2+} antiport. On the other hand, α_1-adrenoceptor stimulation also increases Na^+–K^+ pump activity (Zaza, Kline & Rosen, 1990; Wilde & Kleber, 1991), so that both Na^+ influx and efflux may be enhanced in one or other direction (Terzic *et al.* 1993).

Summarizing all the above-mentioned actions of PKC, this enzyme may regulate inotropy by changing cytosolic Ca^{2+} transients, Ca^{2+} sensitivity of the myofilaments and cytosolic pH. Which of these mechanisms accounts for the observed negative inotropic effect of adrenaline cannot be ascertained from the present experiments. Speculating upon the biological significance of the α_1-adrenoceptor-mediated negative inotropy, it could be suggested that it represents a protective effect against Ca^{2+} overload, comparable to the β-adrenoceptor-mediated effect of cAMP, which not only increases Ca^{2+} transients, but also diminishes the Ca^{2+} sensitivity of troponin.

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Received 26 July 1996; accepted 1 November 1996.